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PRINCIPAL INVESTIGATOR: Michel Roberge

CONTRACTING ORGANIZATION: The University of British Columbia
Vancouver, Canada V6T1Z3

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Michel Roberge
Betty Diamond

E-Mail: michelr@mail.ubc.ca

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14. ABSTRACT

The goal in year 1 of this award was to determine the extent to which six drugs reduce or normalize the pathologically high mTORC1 activity seen in TSC-defective cells, to determine whether they can improve other abnormal phenotypes described for TSC-defective cells, and to select a candidate for proof of concept in animal studies in year 2. The effect of drugs on mTORC1 signaling, cell viability, serum-independent proliferation, p27 nuclear localization and cell motility were determined in $TSC2^{+/+}$ and $TSC2^{-/-}$ cells. Two drugs, nitazoxanide and tizoxanide, did not significantly inhibit mTORC1 in these cells. Three additional drugs, perhexiline, amiodarone and niclosamide appear too toxic to be viable drugs for treatment of tuberous sclerosis. Dronedarone is the only drug with potential for in vivo testing.

15. SUBJECT TERMS
mTOR, dronedarone, p27, cytotoxicity, TSC

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Table of Contents

Introduction

Tuberous sclerosis is a disease associated with abnormally high mTORC1 signaling. The goal of this research is to determine the extent to which five approved drugs and one active drug metabolite that are mTOR signaling inhibitors (niclosamide, perhexiline, amiodarone, dronedarone, nitazoxanide and its active metabolite tizoxanide,) can reduce or normalize the high mTORC1 activity seen in TSC-defective cells and can ameliorate other abnormal phenotypes described for TSC-defective cells, and to select the best drug candidate to determine whether mTORC1 inhibition and inhibition of the growth of TSC-defective cells can be achieved in vivo at tolerated drug doses.

Body

Specific Aim 1.1. Normalization of mTORC1 signaling in TSC-defective cells.

In previous experiments (Balgi, 2009), we had tested the effect of short-term exposure (4 h) to niclosamide, perhexiline, and amiodarone on mTORC1 signaling in $TSC2^{+/+}$ and $TSC2^{-/-}$ cells. The amiodarone analog dronedarone was only approved by the FDA in 2009. We have now compared the effect of amiodarone and dronedarone on mTORC1 activity in $TSC2^{+/+}$ and $TSC2^{-/-}$ cells. Dronedarone effectively reduced mTORC1 signaling at 3 μM and higher concentrations as shown by reduction of the phosphorylation of the mTORC1 substrate S6 kinase at threonine 389, and by its increased electrophoretic mobility (Figure 1). It is considerably more potent than amiodarone, which inhibits mTORC1 signaling in the 10-30 μM concentration range (Figure 1). This observation is relevant because dronedarone appears to be a substantially safer drug than amiodarone in humans.

Nitazoxanide and tizoxanide inhibit mTORC1 signaling in MCF-7 human breast cancer cells and THP1 human leukemia cells (Lam, 2012). We have tested them on $TSC2^{+/+}$ and $TSC2^{-/-}$ mouse embryo fibroblasts. Neither drug showed significant inhibition of mTORC1 signaling in these cells (Figure 2). Nitazoxanide and tizoxanide were therefore excluded as potential therapeutics for tuberous sclerosis and they were not studied further.

We next tested the effect of niclosamide, perhexiline, amiodarone and dronedarone on mTORC1 signaling during long-term exposure (72h). By 72 h, mTORC1 signaling had returned to untreated levels for all drugs unlike rapamycin, an irreversible mTORC1 inhibitor used as a positive control in these experiments (Figure 3). The results indicate that mTORC1 inhibition by these four drugs is reversible and that the drugs may be metabolically inactivated during long term exposure.

We next determined the effects of the drugs on cell viability. There was no significant reduction in cell viability during 4 h exposure to the drugs except at the highest concentrations of perhexiline and dronedarone (10 μM; Figure 4).

Therefore, during 4 h exposure, niclosamide, amiodarone and dronedarone significantly inhibited mTORC1 signaling in $TSC2^{-/-}$ cells at concentrations that are not toxic (10 μM niclosamide, 30 μM amiodarone and 3 μM dronedarone). By contrast, perhexiline was toxic to cells at the concentration that inhibits mTORC1 signaling (10 μM), indicating that it is less promising as a drug candidate for treatment of tuberous sclerosis.

The effect of the drugs on cell viability was also measured during long term exposure (5 days). Both $TSC2^{+/+}$ and $TSC2^{-/-}$ cells showed reduced viability compared with untreated controls following treatment with niclosamide above 0.3 μM, perhexiline above 3 μM, amiodarone above 1 μM and dronedarone above 1 μM (Figures 5 and 6). Combined, these results indicate that the drugs do not kill cells but reduce their proliferation, which is expected of mTORC1 inhibitory drugs.

Specific Aim 1.2. Amelioration of abnormal phenotypes associated with the TSC defect.

A number of morphological and biochemical abnormalities have been reported in cultured TSC-defective cells. We examined whether the four drugs could ameliorate any of these abnormalities.

1- serum-independent proliferation
It has been reported that $TSC2^{-/-}$ cells are able to proliferate in medium lacking serum while $TSC2^{+/+}$ cells are unable to do so. We observe that $TSC2^{+/+}$ cells are more dependent on serum for proliferation than $TSC2^{-/-}$ cells (Figure 7). However, the proliferation of $TSC2^{-/-}$ cells was not completely independent of serum, rather the cells proliferated at a lower rate in the absence of serum than in its presence (Figure 7).

To determine whether the drugs reduced this difference and made $TSC2^{-/-}$ cell proliferation more sensitive to serum withdrawal, we treated $TSC2^{+/+}$ cells and $TSC2^{-/-}$ cells with the drugs in the absence or presence of serum and viable cells were measured at 48 h (Figures 8 and 9). The decreased sensitivity of $TSC2^{-/-}$ cells to serum withdrawal was not corrected by exposure to niclosamide (Figure 8) at low concentrations that did not reduce the viability or proliferation of both cell lines. Higher concentrations of niclosamide caused reduced viability in both cell lines in serum and without serum. The other drugs also did not correct this defect (Figure 9).

2-reduced p27 expression and nuclear localization
It has been reported that TSC2-defective fibroblasts have reduced levels of the protein p27 and mislocalization of p27 from the nucleus to the cytoplasm. We used quantitative automated immunofluorescence microscopy to examine whether the drugs cause increased localization of p27 to the nucleus. Cells were treated with the drugs for 4 h, after which they were fixed with paraformaldehyde and incubated with p27 antibody followed by a fluorescently labeled secondary antibody. In the absence of drug treatment, p27 was localized to the cytoplasm and the nucleus in both cell lines (Figure 10).

To provide a quantitative measure, nuclear fluorescence intensity was determined in many cells in duplicate wells. Table 1 shows the average p27 fluorescence intensity (arbitrary fluorescence units) and the number of nuclei measured for each well and condition. Niclosamide caused an increase in nuclear p27 but this increase was more pronounced in $TSC2^{+/+}$ cells than in $TSC2^{-/-}$ cells. Dronedarone caused an increase in nuclear p27 in both $TSC2^{-/-}$ and $TSC2^{+/+}$ cells, and only at high concentrations that reduce cell viability. Amiodarone has not been tested.

3- reduced cell motility

TSC2-defective cells are reported to have reduced motility. We have used two assays to measure the effect of drugs on cell motility: a wound healing assay and a fluorescent bead track clearing assay. Both assays confirmed previous reports that TSC2-defective cells have reduced motility. In the wound healing assay, cells were cleared from a confluent monolayer and the width of the cleared zone (the wound) was measured over time. 5.5 h after wound generation $TSC2^{+/+}$ cells had migrated into the gap left by the wound more than $TSC2^{-/-}$ cells (Figures 11, 12, 13). In the fluorescent bead track clearing assay, a uniform lawn of small fluorescent beads is deposited onto a cell culture plastic surface. Cells are then added and as they move along the surface, they phagocytose the beads, leaving a non-fluorescent track whose area can be measured by automated fluorescence microscopy. The tracks of $TSC2^{-/-}$ cells were smaller than those of $TSC2^{+/+}$ cells (Figures 14, 15 and 16).

In the wound healing assay, drugs were added and the width of the cleared zone (the wound) was measured over time. In both $TSC2^{+/+}$ and $TSC2^{-/-}$ cells, perhexiline, niclosamide and dronedarone did not affect wound healing but amiodarone significantly inhibited wound healing (Figures 12 and 13)

In the bead clearing assay, the drugs had little or no effect on cell motility of either cell line except for nitazoxanide (10 μM) which increased the motility of $TSC2^{-/-}$ cells (Figures 14, 15 and 16).

4- membrane ruffling defects

Membrane ruffling is a process that occurs at the leading edge of cells while they migrate. $TSC2^{-/-}$ cells have been reported to display reduced ruffling activity. Membrane ruffling is observed under the light microscope and is not a quantitative assay. Its main purpose here was to confirm results obtained in the cell motility assays. However, none of the drugs increased the migration of $TSC2^{-/-}$ cells in both the wound healing and the bead clearing assays. For this reason, the effects of the compounds on membrane ruffling were not tested.

5- Reduced PDGF-PI3K-Akt signaling

This task has not yet been carried out.

In summary, although niclosamide, perhexiline, amiodarone and dronedarone were shown to inhibit mTORC1 signaling in $TSC2^{-/-}$ cells, none clearly ameliorates the phenotypic defects displayed by $TSC2^{-/-}$ cells. Two drugs, nitazoxanide and its active metabolite tizoxanide did not significantly inhibit mTORC1 signaling. Two drugs, perhexiline and amiodarone, inhibited mTORC1 signaling in $TSC2^{-/-}$ cells but they appear too toxic to be viable drug candidates for treatment of tuberous sclerosis. Niclosamide inhibited mTORC1 signaling in $TSC2^{-/-}$ cells. However, we have recently defined its mechanism of action: it inhibits mTORC1 by protonophoric activity (Fonseca et al., J. Biol. Chem. 287:17530, 2012). Protonophores are known to cause deleterious effects such as plasma membrane depolarization and mitochondrial uncoupling, making them unattractive as therapeutic agents. Dronedarone is therefore the only drug with potential for in vivo testing in Specific Aim 2.

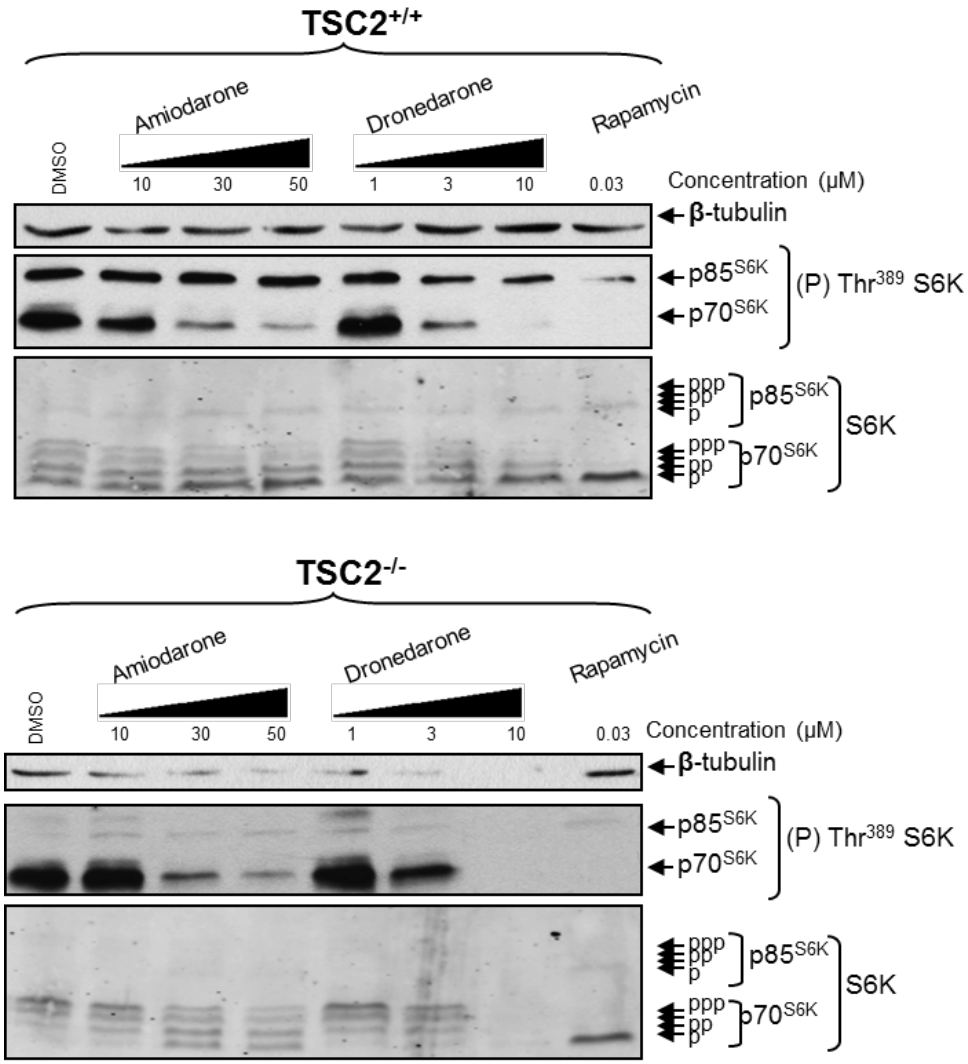


Figure 1. Effect of amiodarone and dronedarone on mTORC1 signaling in $TSC2^{+/+}$ and $TSC2^{-/-}$ cells. The cells were incubated for a short period of time (4 h) with the indicated concentrations of drugs and the phosphorylation of the mTORC1 substrate S6K was measured by western blotting.This experiment was carried out three times, with similar results.

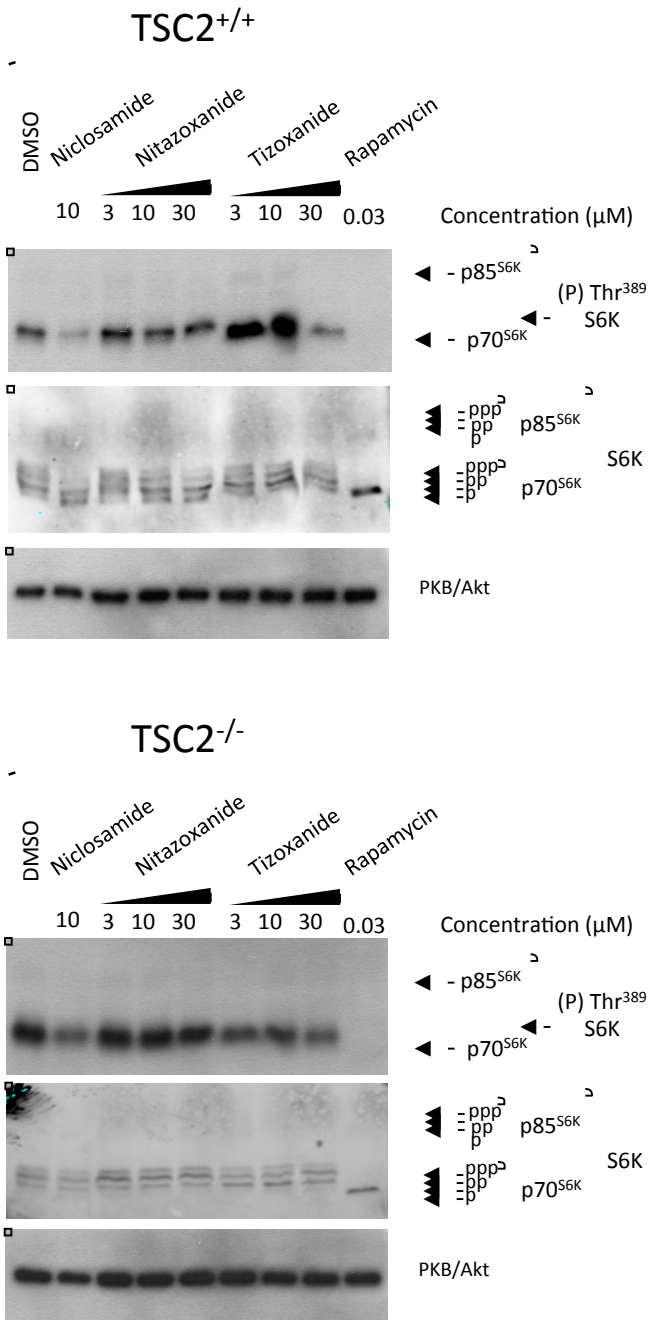


Figure 2. Effect of nitazoxanide and tizoxanide on mTORC1 signaling in $TSC2^{+/+}$ and $TSC2^{-/-}$ cells. The cells were incubated for a short period of time (4 h) with the indicated concentrations of drugs and the phosphorylation of the mTORC1 substrate S6K was measured by western blotting. This experiment was carried out twice, with similar results.

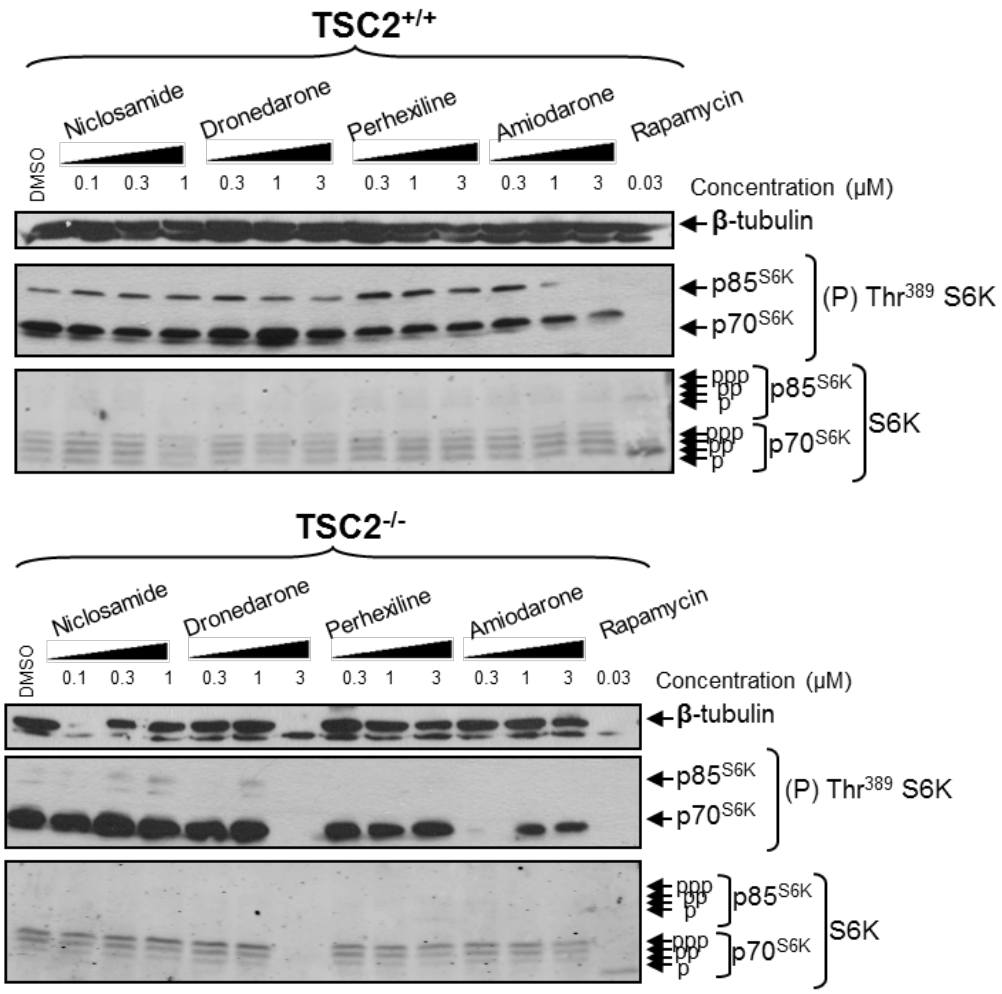


Figure 3. Effect of drugs on mTORC1 signaling in $TSC2^{+/+}$ and $TSC2^{-/-}$ cells. The cells were incubated for a long period of time (72 h) with the indicated concentrations of niclosamide, dronedarone, perhexiline, amiodarone or rapamycin, and the phosphorylation of the mTORC1 substrate S6K was measured by western blotting. This experiment was carried out twice, with similar results.

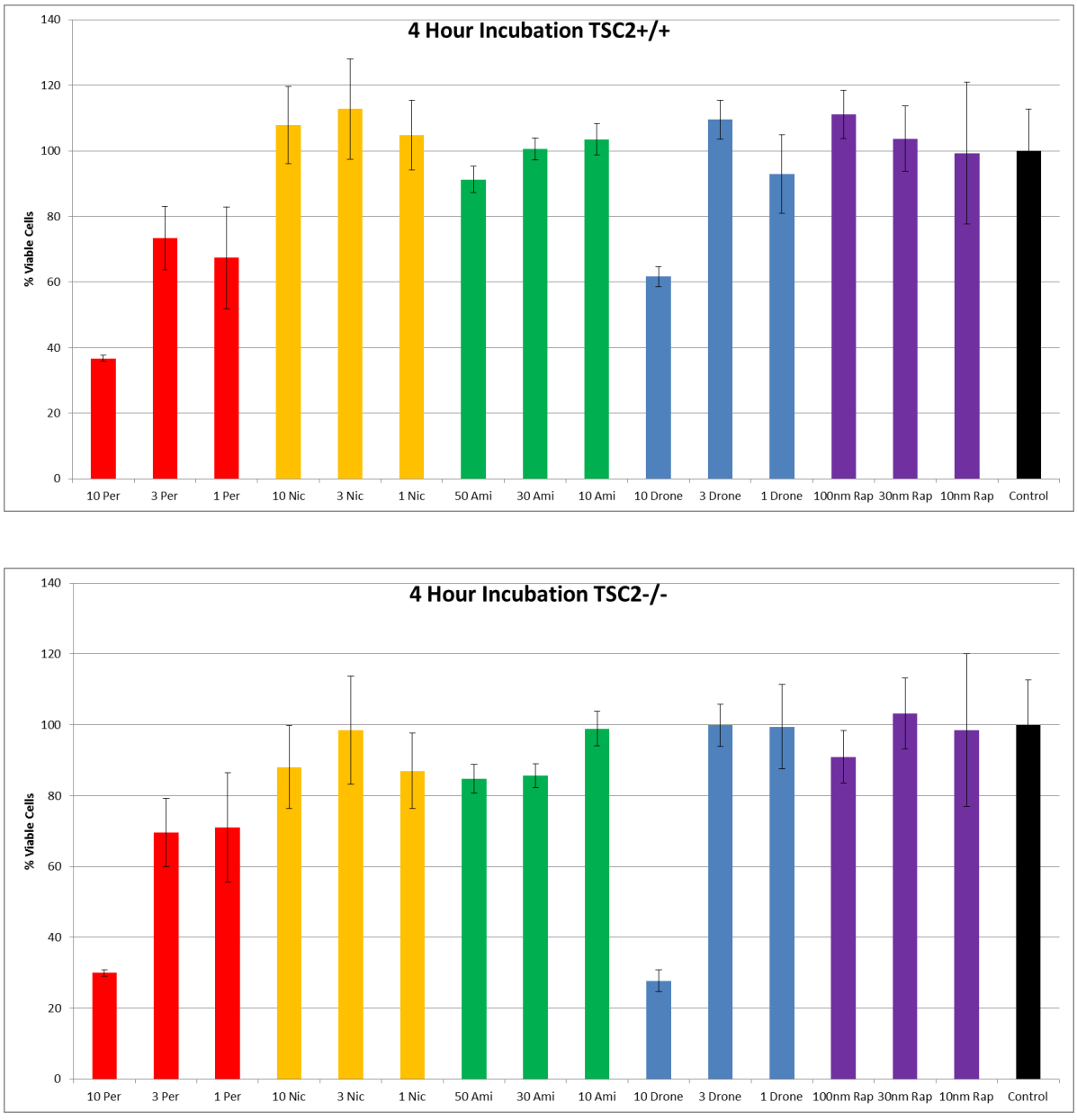
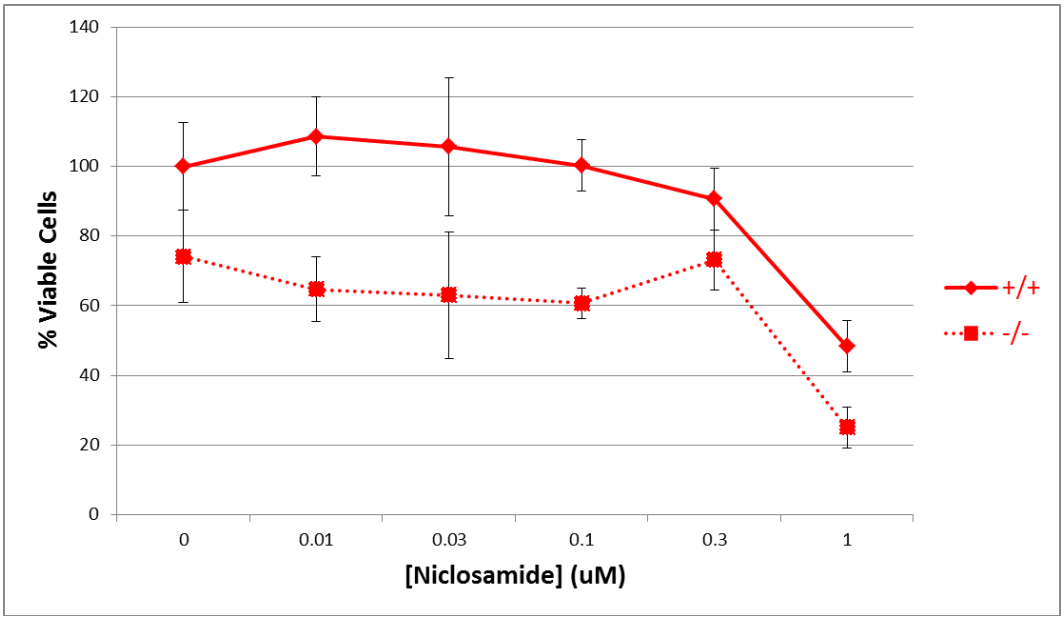


Figure 4. Effect of drugs on cell viability. $TSC2^{+/+}$ and $TSC2^{-/-}$ cells were incubated for 4 hours with the indicated micromolar concentrations of perhexiline (Per), niclosamide (Nic), amiodarore (Ami), dronedarone (Drone), rapamycin (Rap), and viable cell numbers were determined. Error bars represent positive and negative values of one standard deviation (N=3).



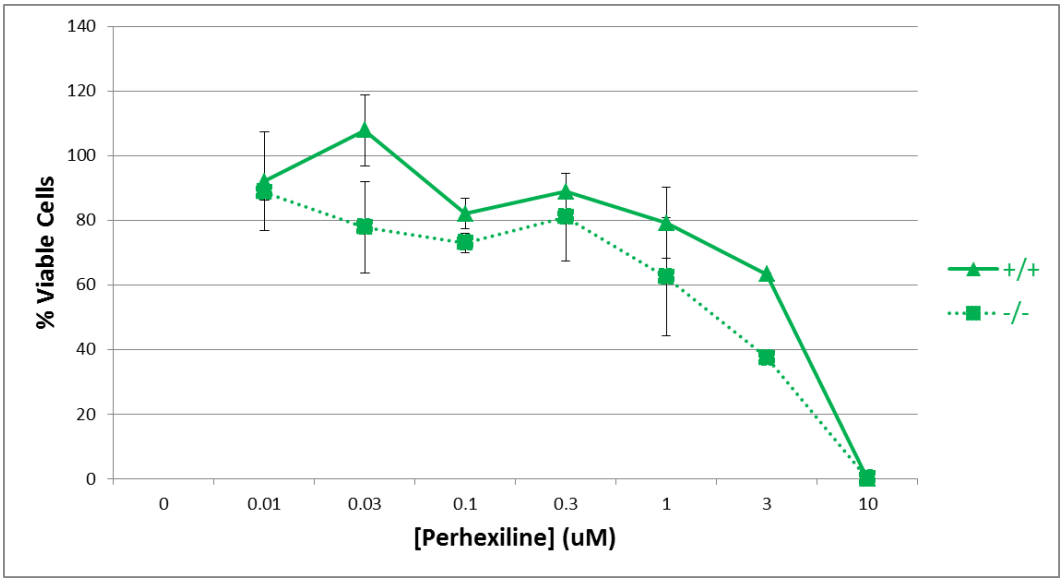
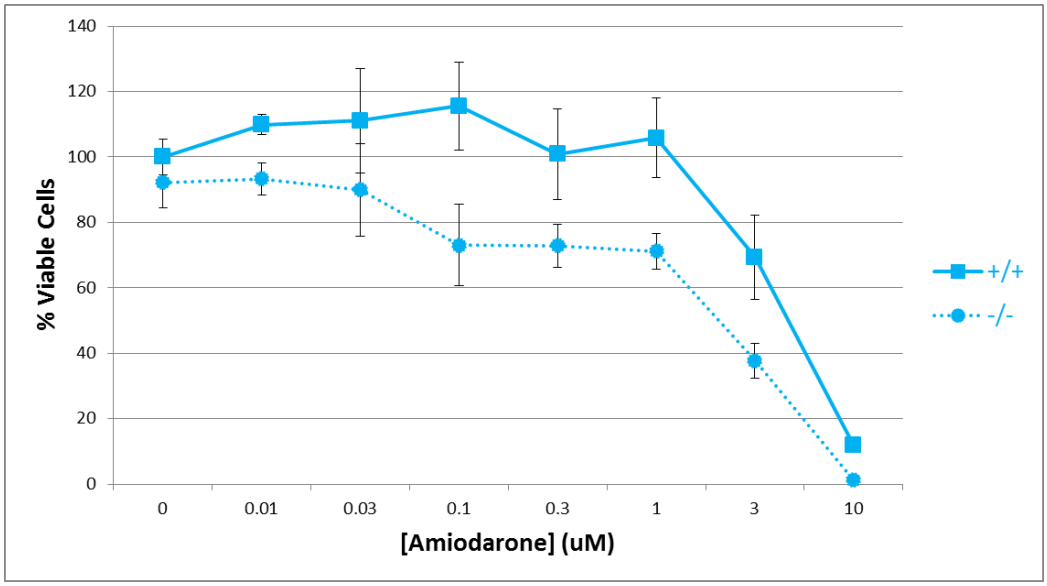


Figure 5. Effect of drugs on cell viability. $TSC2^{+/+}$ and $TSC2^{-/-}$ cells were incubated with the indicated micromolar concentrations of niclosamide or perhexiline and cell viability was measured after 5 days using the MTT assay. Error bars represent positive and negative values of one standard deviation (N=3).



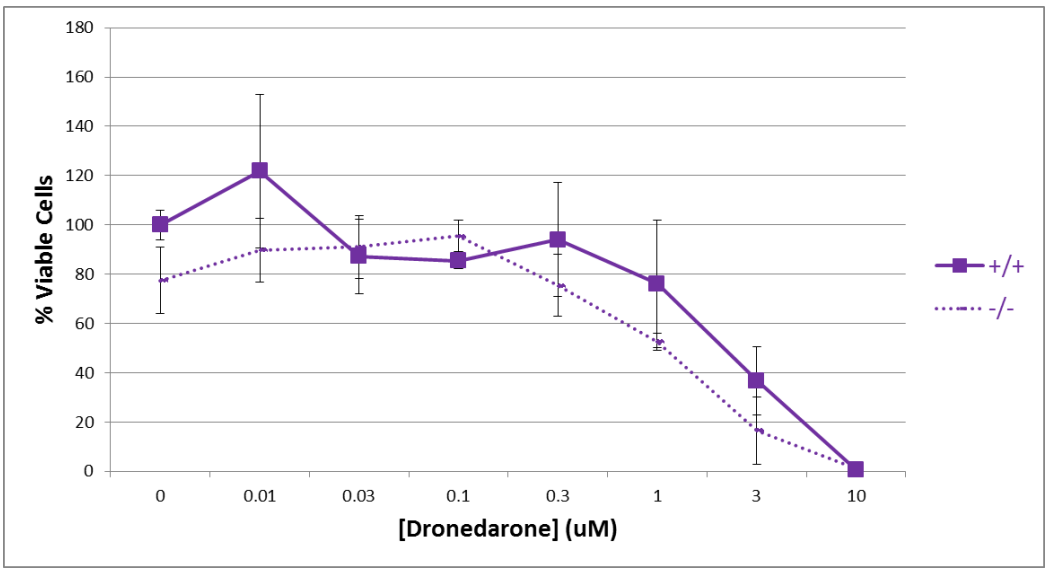


Figure 6. Effect of drugs on cell viability. $TSC2^{+/+}$ and $TSC2^{-/-}$ cells were incubated with the indicated micromolar concentrations of amiodarone or dronedarone and cell viability was measured after 5 days using the MTT assay. Error bars represent positive and negative values of one standard deviation (N=3).

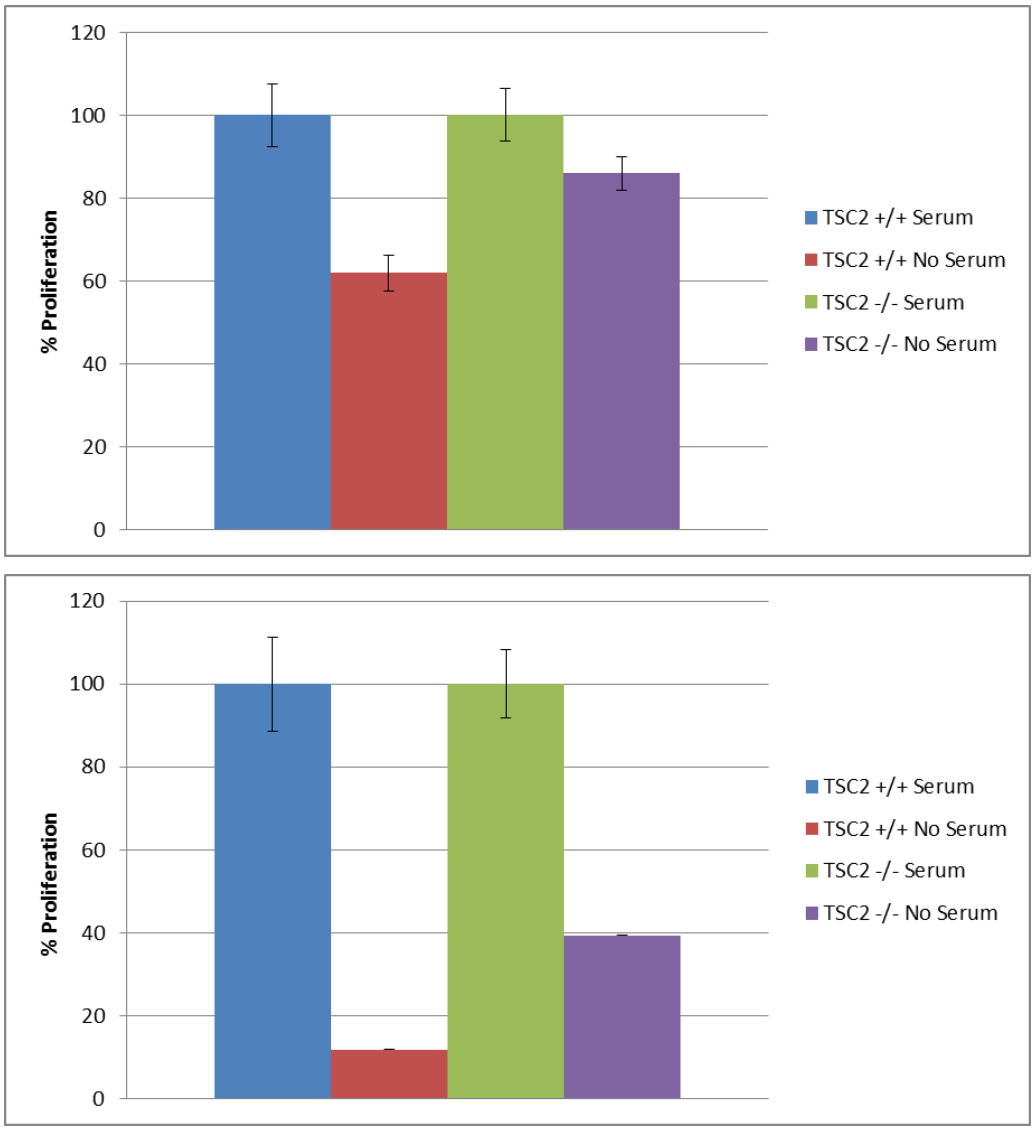


Figure 7. Effect of serum deprivation on the proliferation of $TSC2^{+/+}$ and $TSC2^{-/-}$ cells. Cells were exposed to culture medium containing 10% serum (Serum) or 0% serum (No Serum) for 24 h (upper panel) or 48 h (lower panel) and cell viability was determined using the MTT assay. Error bars represent positive and negative values of one standard deviation (N=3).

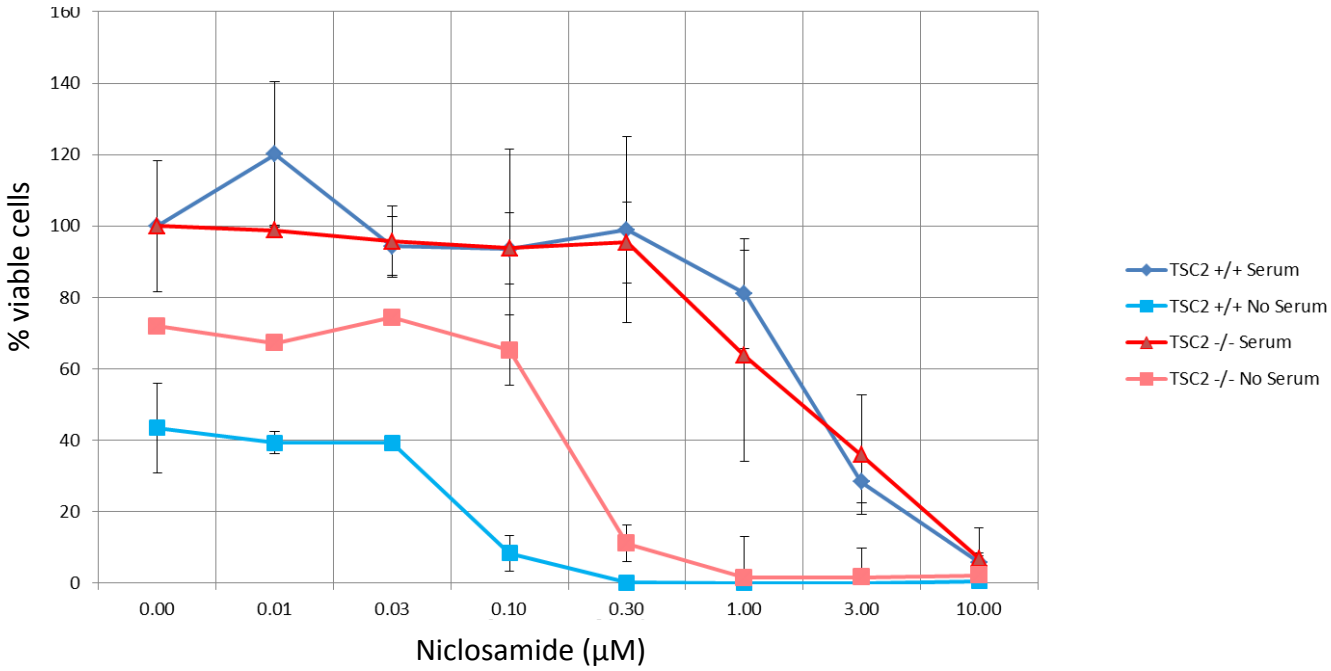


Figure 8. Effect of niclosamide on cell proliferation in the presence or absence of serum. $TSC2^{+/+}$ and $TSC2^{-/-}$ cells were exposed to the indicated concentrations of niclosamide for 48 h in medium containing 10% fetal bovine serum (Serum) or 0% serum (No Serum). Cell viability was measured using the MTT assay. Error bars represent positive and negative values of one standard deviation (N=3).

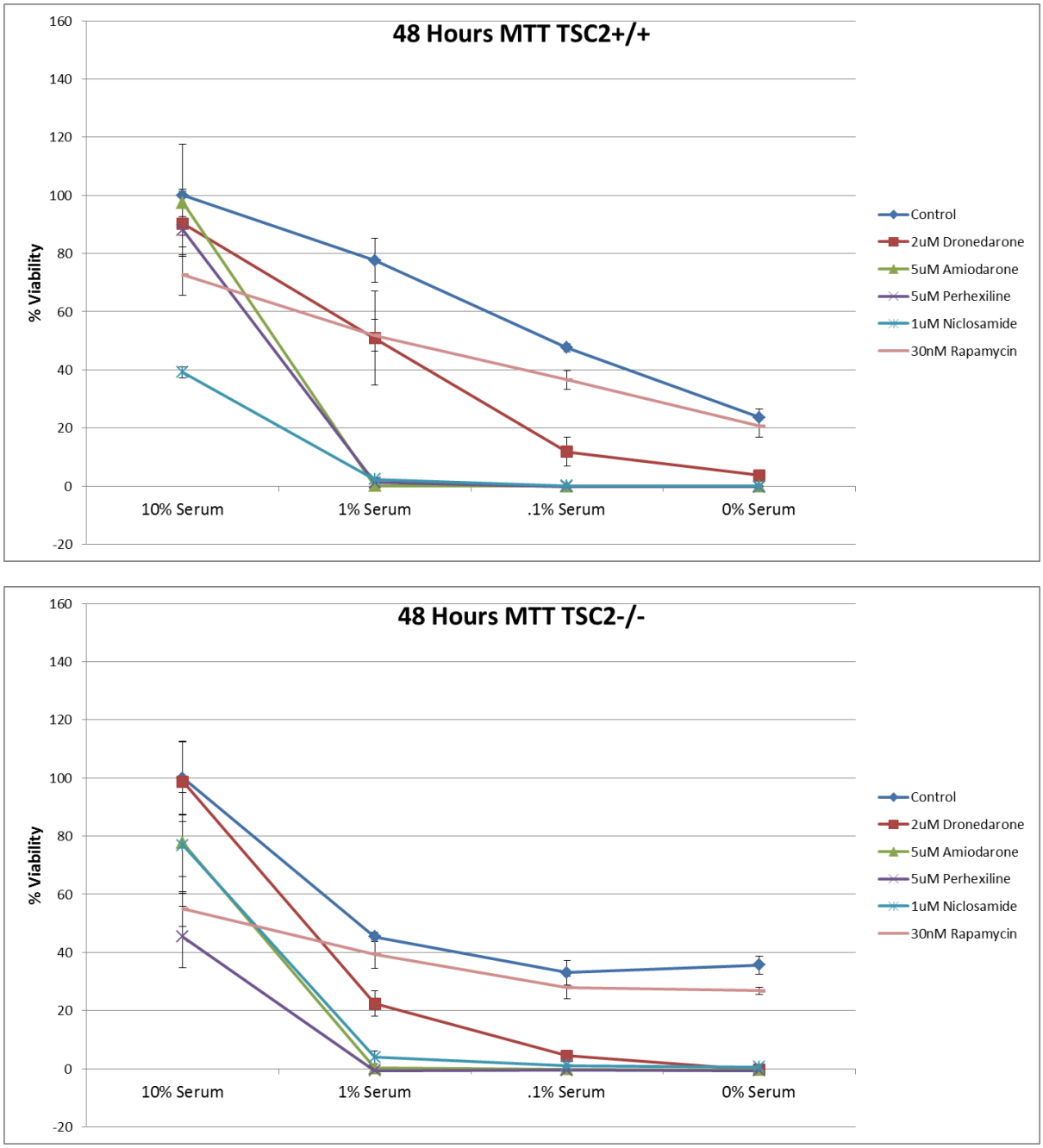


Figure 9. Effect of additional drugs on cell proliferation in the presence or absence of serum. $TSC2^{+/+}$ and $TSC2^{-/-}$ cells were exposed to the indicated concentrations of drugs for 48 h in medium containing different concentrations of fetal bovine serum (Serum). Cell viability was measured using the MTT assay. Error bars represent positive and negative values of one standard deviation (N=3).

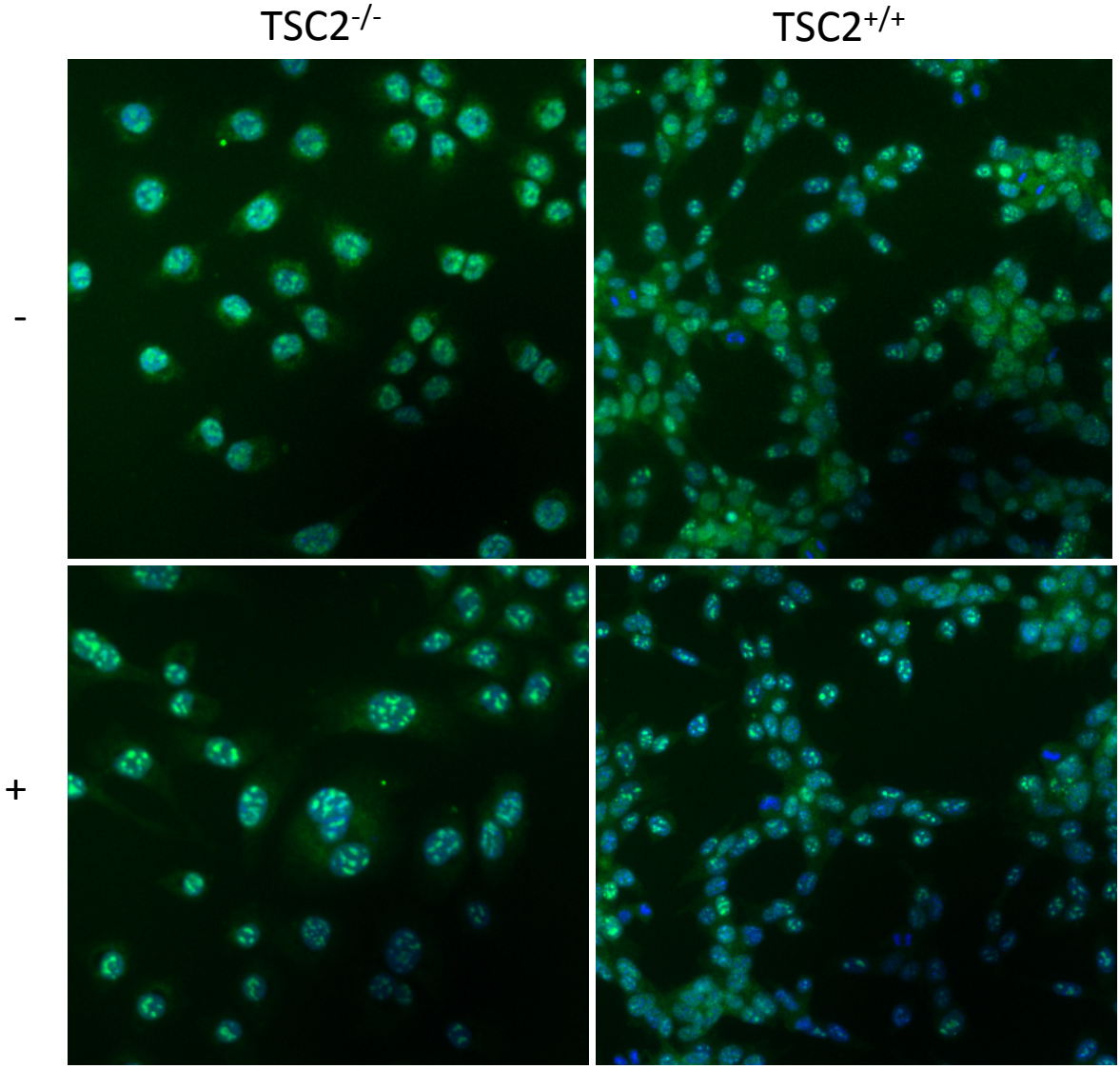


Figure 10. Effect of dronedarone on the intracellular localization of p27. $TSC2^{+/+}$ and $TSC2^{-/-}$ cells were exposed to 0 μM dronedarone (-) or 2 μM dronedarone (+) dronedarone for 4 h and p27 was detected by immunofluorescence microscopy (green). The cells were also stained with the DNA dye Hoechst 33342 to visualize nuclei (blue).

Table 1 Effect of drugs on nuclear p27 localization

	nuclear p27					Dronedarone						
	No drug	nic, 3μM	nic, 10μM	rapa, 30nM	rapa, 100nM	0.5 μM	1μM	2 μM	4μM	6 μM	8 μM	10μM
TSC2 +/+	259	605	815	244	215	267	287	351	349	441	801	289
	272	646	761	211	224	272	284	310	283	373	801	880
avg	**265**	**626**	**788**	**228**	**220**	**269**	**286**	**330**	**316**	**407**	**801**	**585**

	number of nuclei					Dronedarone						
	No drug	nic, 3μM	nic, 10μM	rapa, 30nM	rapa, 100nM	0.5 μM	1μM	2 μM	4μM	6 μM	8 μM	10μM
TSC2 +/+	2874	1585	1281	3793	3782	3447	3354	3101	3372	2406	1069	34
	3223	1817	1479	4209	3551	4014	3466	3442	3654	2953	1682	276
avg	**3049**	**1701**	**1380**	**4001**	**3667**	**3731**	**3410**	**3272**	**3513**	**2680**	**1376**	**155**

	nuclear p27					Dronedarone						
	No drug	nic, 3μM	nic, 10μM	rapa, 30nM	rapa, 100nM	0.5 μM	1μM	2 μM	4μM	6 μM	8 μM	10μM
TSC2 -/-	536	685	824	603	542	465	543	539	633	968	610	82
	661	860	948	612	591	594	547	635	690	762	829	31
avg	**598**	**772**	**886**	**607**	**566**	**529**	**545**	**587**	**662**	**865**	**720**	**56**

	number of nuclei					Dronedarone						
	No drug	nic, 3μM	nic, 10uM	rapa, 30nM	rapa, 100nM	0.5 μM	1μM	2 μM	4μM	6 μM	8 μM	10μM
TSC2 -/-	398	252	191	395	370	676	648	492	422	123	147	346
	399	222	192	437	417	498	589	646	349	328	270	123
avg	**399**	**237**	**192**	**416**	**394**	**587**	**619**	**569**	**386**	**226**	**209**	**235**

Nuclear p27 numbers are arbitrary fluorescence intensity numbers in the nuclear region defined by DNA staining.

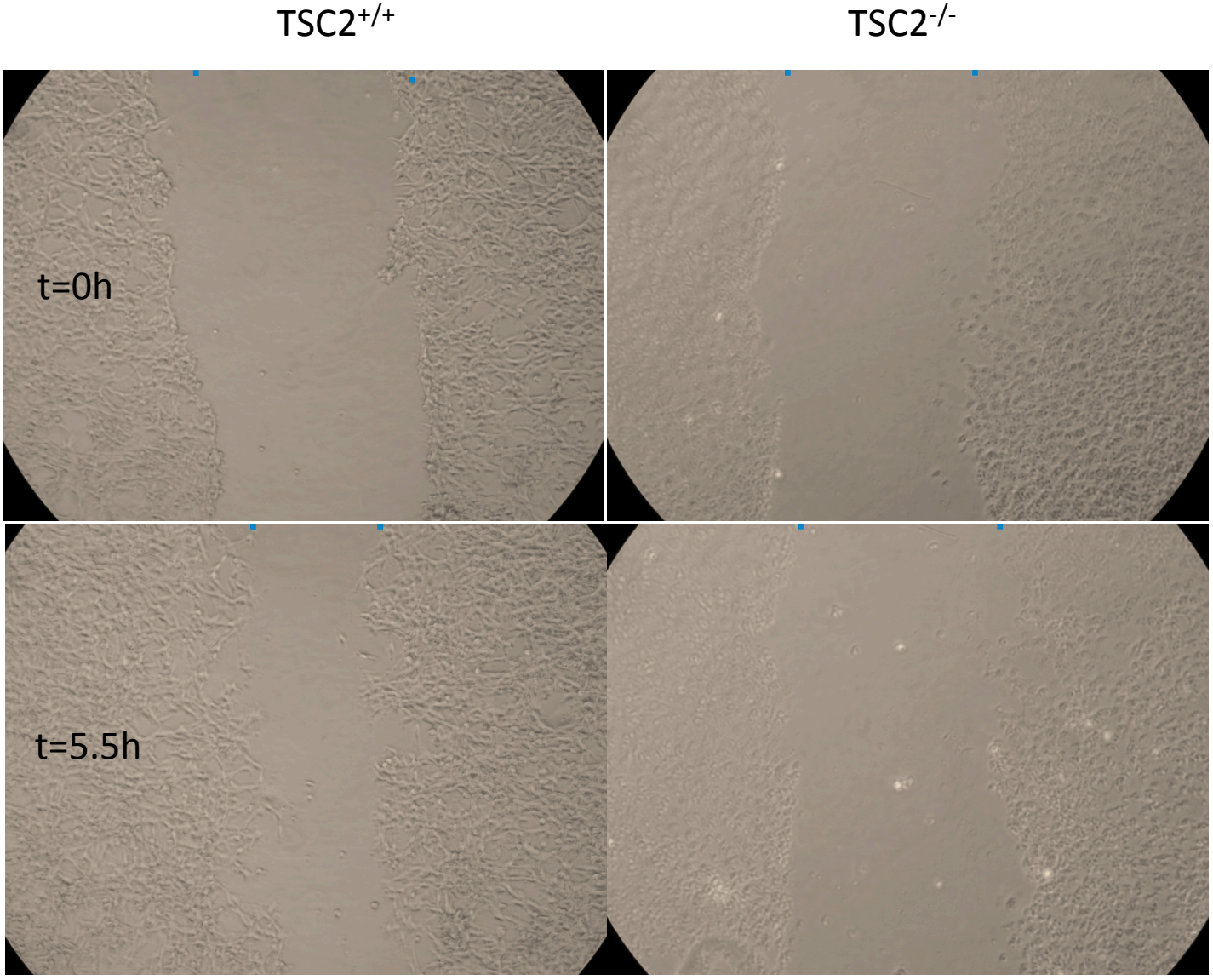
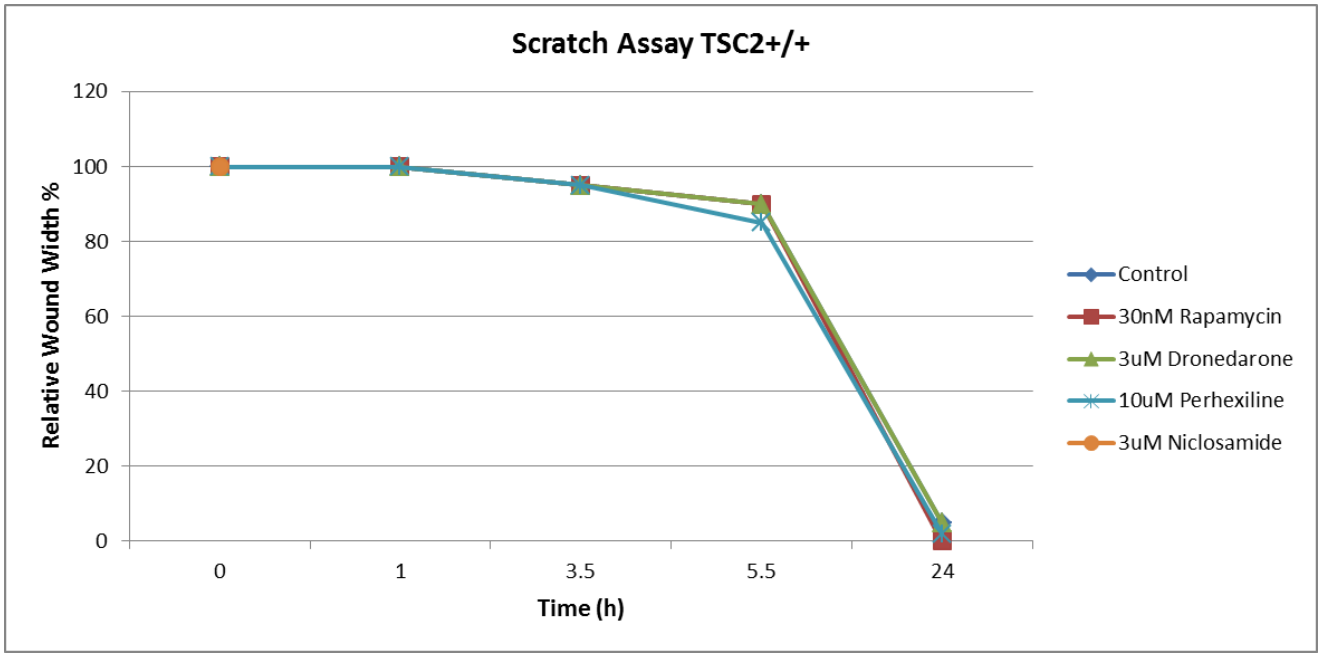


Figure 11. Wound healing assay used to examine the effect of drugs on cell motility. Confluent $TSC2^{+/+}$ and $TSC2^{-/-}$ cells monolayers were scratched to generate a gap (t=0h) and cell movement to fill the gap was monitored at t=5h in the same area. The blue bars indicate the gap boundaries.



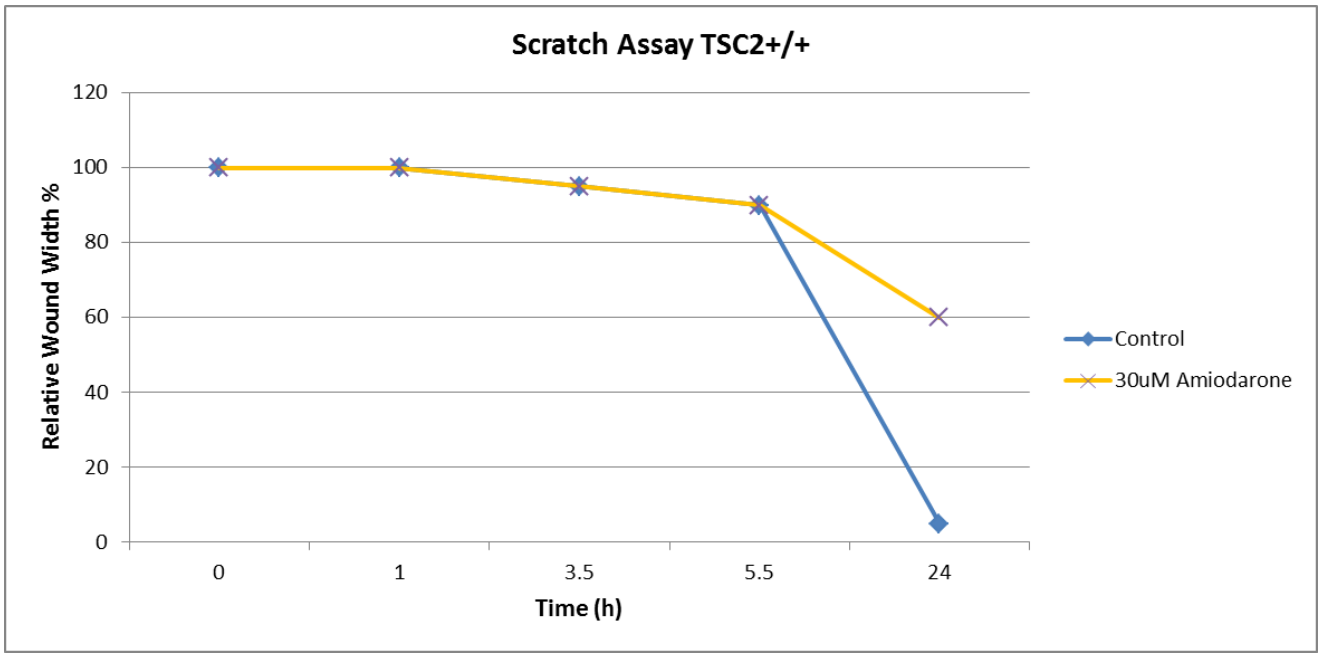
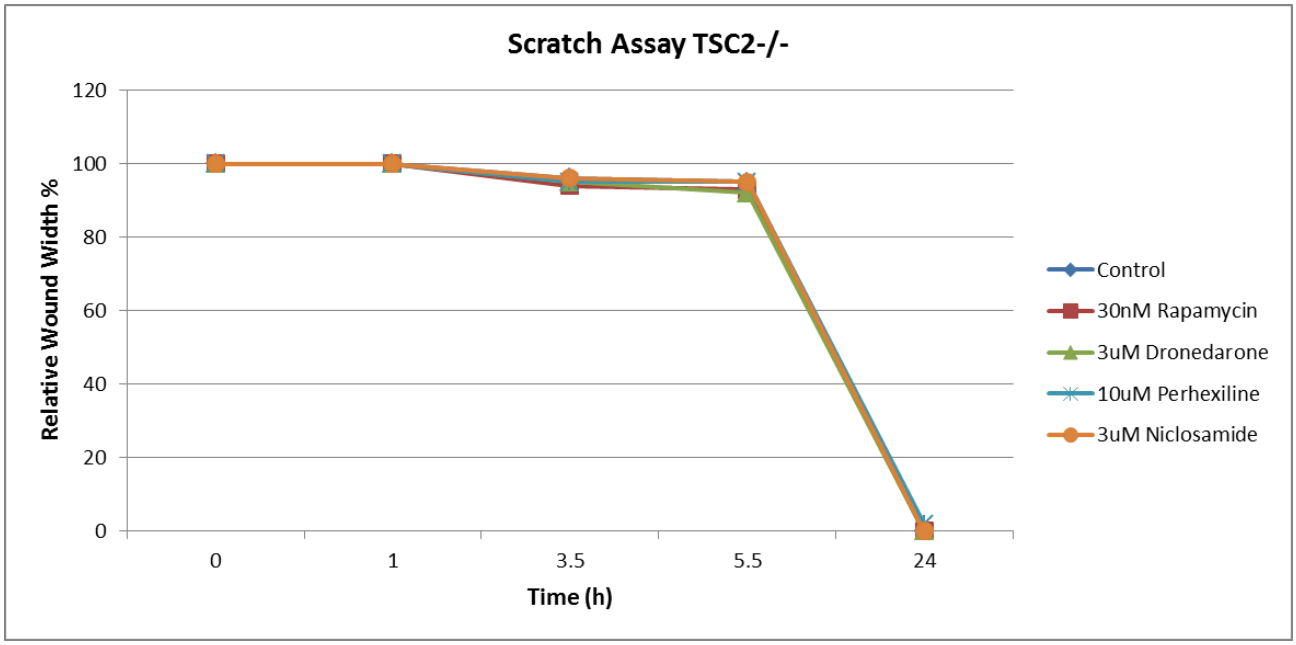


Figure 12. Effect of drugs on wound healing in $TSC2^{+/+}$ cells. Cell migration into the cleared area was measured over time after addition of drugs t=0h.



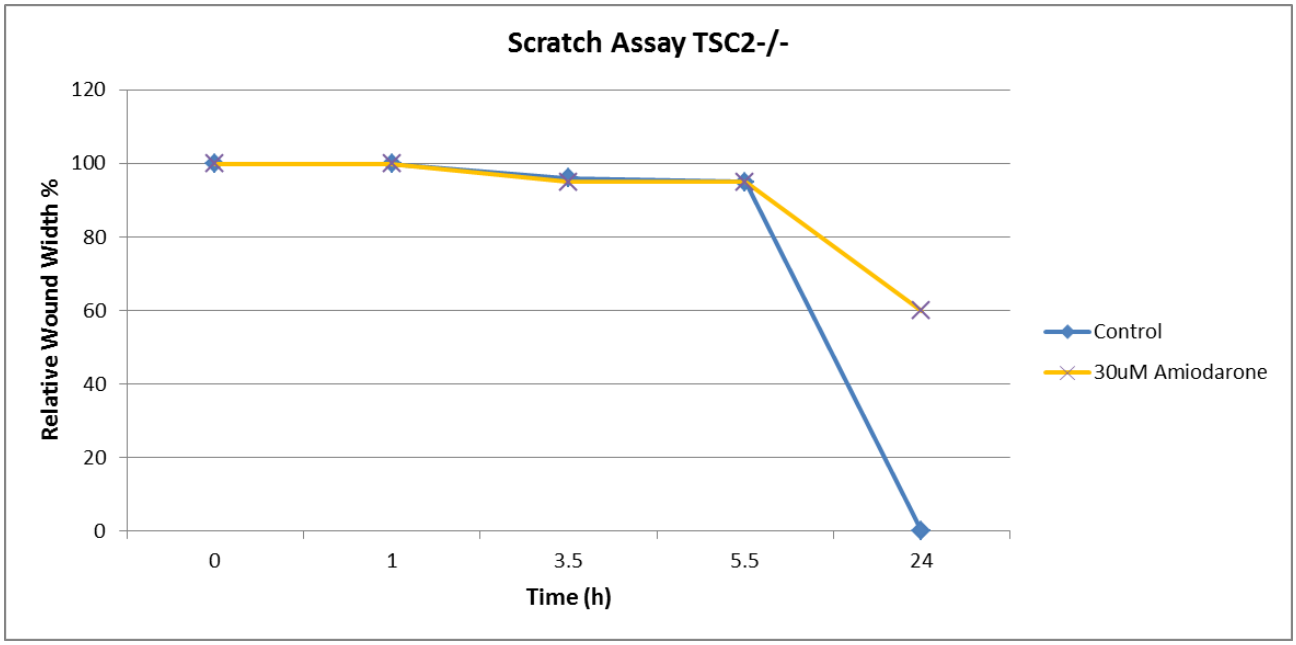


Figure 13. Effect of drugs on wound healing in TSC2$^{-/-}$ cells. Cell migration into the cleared area was measured over time after addition of drugs t=0h.

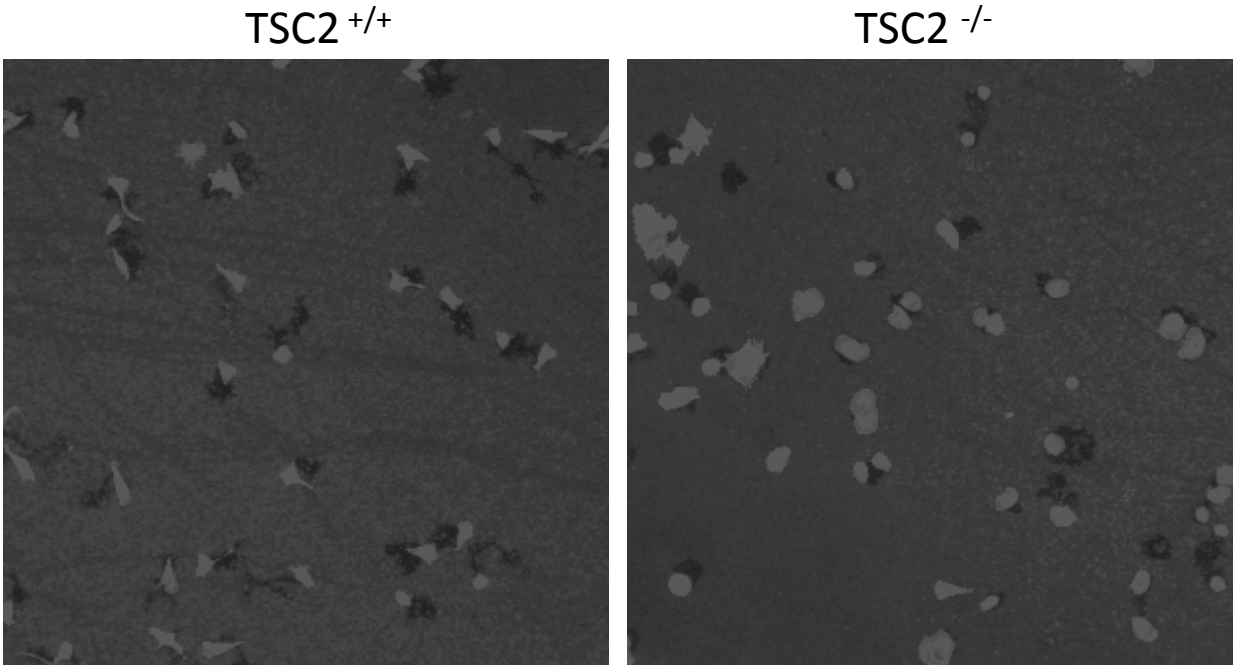


Figure 14. Fluorescent bead clearing track clearing assay used to examine the effect of drugs on cell motility. $TSC2^{+/+}$ and $TSC2^{-/-}$ cells were deposited onto a lawn of fluorescent beads. As cells moved, they phagocytosed the beads, leaving a fluorescence-free track. After 5 hours, the cells were also stained with a fluorescent dye. The area cleared of fluorescent beads was measured by automated fluorescence microscopy.

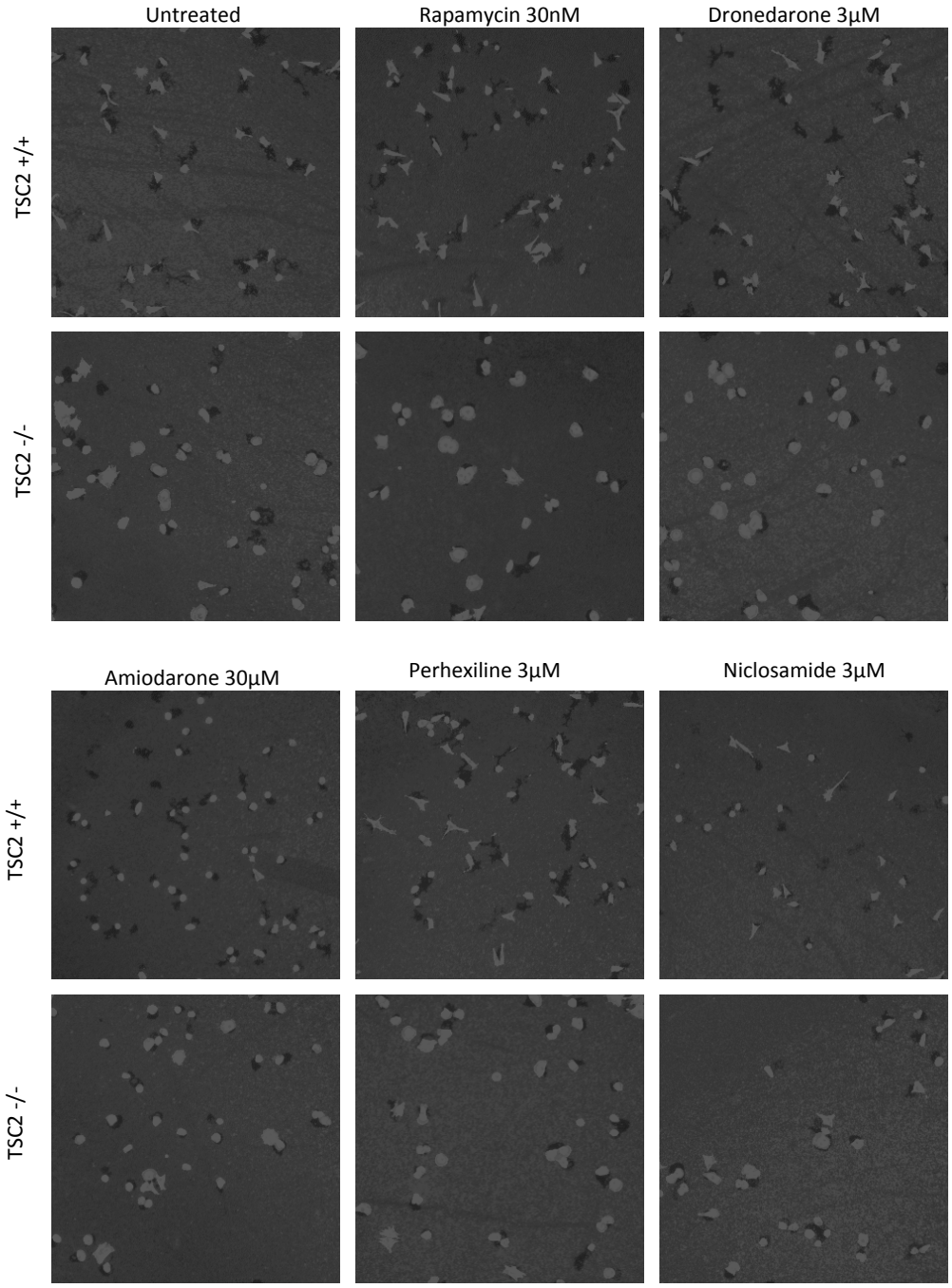


Figure 15. Effect of drugs on fluorescent bead clearing. $TSC2^{+/+}$ and $TSC2^{-/-}$ cells were deposited onto a lawn of fluorescent beads and drugs were added for 5 hours. The area cleared of fluorescent beads was measured by automated fluorescence microscopy.

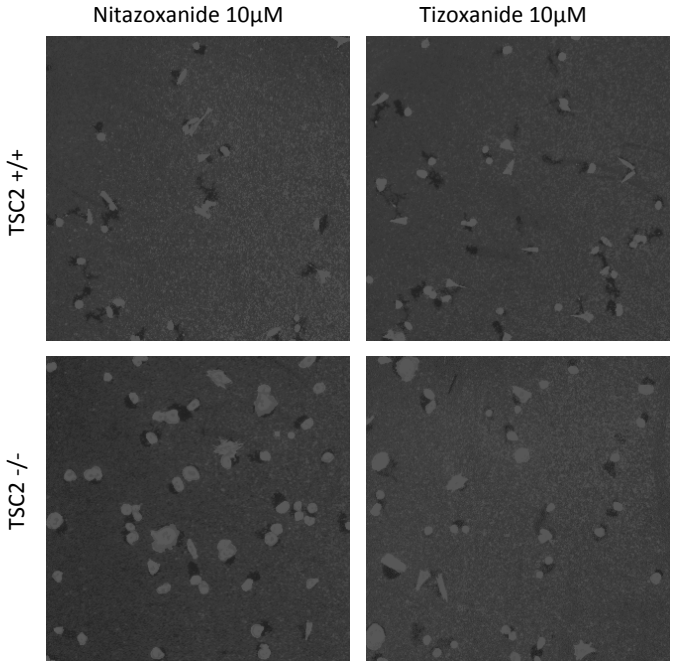


Figure 15 (continued).

Mean Motion Trac kArea

	TSC2 +/+			TSC2 -/-		
	Untreated	Rapamycin 3 nM	Rapamycin 30 nM	Untreated	Rapamycin 3 nM	Rapamycin 30 nM
	5085	3307.29	3435.38	2958.03	2253.9	2296.35
	2770.1	4104.81	3152.21	4323.79	2108.7	1842.03
Average	3927.55	3706.05	3293.80	3640.91	2181.30	2069.19
StdDev	1636.88	563.93	200.23	965.74	102.67	321.25

Mean Motion Trac kArea

	Untreated	Dronedarone 3 µM	Amiodarone 30 µM	Perhexiline 3 µM	Niclosamide 3 µM	Nitazoxanide 10 µM	Tizoxanide 10 µM
TSC2 +/+	5810.04	5535.54	4426.19	4797.36	3607.18	6127.42	5410.41
	6379.52	6847.07	3472.17	4354.51	3601.54	6871.4	
Average	6094.78	6191.31	3949.18	4575.94	3604.36	6499.41	5410.41
StdDev	402.68	927.39	674.59	313.14	3.99	526.07	0.00

	Untreated	Dronedarone 3 µM	Amiodarone 30 µM	Perhexiline 3 µM	Niclosamide 3 µM	Nitazoxanide 10 µM	Tizoxanide 10 µM
TSC2 -/-	4442.67	3741.76	3245.58	4948.39	4537	6457.65	4829.96
	4453.42	3512.36	3747.14	3920.24	4474.66	5900.65	
Average	4448.05	3627.06	3496.36	4434.32	4505.83	6179.15	4829.96
StdDev	7.60	162.21	354.66	727.01	44.08	393.86	0.00

Figure 16. Effect of drugs on fluorescent bead clearing. The area cleared of fluorescent beads (Mean Motion Track Area, arbitrary units) was measured by automated microscopy from duplicate samples.

Key research accomplishments

- Identification of dronedarone as a candidate for in vivo studies described in Specific Aim 2, to be carried out in year 2 of this award.

Reportable outcomes

None

Conclusions

In year 1, the goal of Specific Aim 1 to test drugs in in cell culture in vitro to identify the best candidate for in vivo experiments was successfully achieved. Dronedarone was selected. It appears to be a promising candidate as it can inhibit mTORC1 signaling and increase nuclear localization of p27 at a concentration of 2 μM (1.1 μg/ml) in vitro, well within the 0.01 – 5 μg/ml plasma concentrations observed during treatment of humans, and 0.02 – 500 μg/ml observed in tissue recovered from treated animals (Benaim, 2012).

References

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Appendices

none